

METAL REGULATION AND MOLTING IN THE BLUE CRAB, *CALLINECTES SAPIDUS*: COPPER, ZINC, AND METALLOTHIONEIN

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ABSTRACT

Blue crab, *Callinectes sapidus*, hemolymph and digestive glands were examined at different stages of the molt cycle to determine whether molting affected tissue and cytosolic partitioning of copper and zinc and, if so, whether metallothionein was involved. The crabs used in these determinations were not exposed to elevated concentrations of copper or zinc. Concentrations of hemocyanin, copper, and zinc in the hemolymph all decreased significantly during molt. They were lowest at the soft crab stage (A_2) and highest during premolt (D_1 - D_3) and intermolt (C_4). The digestive gland copper concentrations also were highest during premolt (D_1 - D_3) and lowest in the papershell stage (B_1). Zinc followed the same general pattern in both hemolymph and digestive glands. The cytosolic distributions of copper and zinc were determined in the digestive glands using gel filtration chromatography. The elution profiles showed that the percentages of copper and zinc on metallothionein ranged from 10% copper/90% zinc at D_1 to 100% copper at B_1 . The estimated concentrations of metallothionein were highest during intermolt (C_1) and premolt (D_1 - D_3) and lowest during the papershell (B_1). The observed changes in the tissue and cytosolic partitioning of copper and zinc are consistent with the physiological changes occurring in the crabs. These observations support the hypothesis that metallothioneins are naturally occurring proteins that are actively involved in the synthesis of hemocyanin and zinc regulation during the normal processes of growth in blue crabs.

INTRODUCTION

Molting in crustaceans and the biochemical mechanisms that control the turnover and synthesis of metallothioneins are complex and possibly interacting physiological and biochemical processes. Before this investigation, no attempt had been made to link these two physiological activities. This effort, therefore, was designed to determine if changes in the metal composition and amount of metallothionein in the blue crab, *Callinectes sapidus*, are correlated with the molt cycle.

Molting has been examined in detail in a variety of marine and freshwater crustaceans. Skinner (1985) provides an overview of the complex physiological and biochemical processes that are involved in crustacean molting and regeneration with an emphasis on hormonal control. Recently, investigators demonstrated that there are changes in hemocyanin concentrations in the hemolymph of lobsters and blue crabs (Hagerman, 1983; Mangum *et al.*, 1985) during the molt cycle and also osmoregulatory and biochemical changes in molting blue crabs (Towle and Mangum, 1985; Wheatly, 1985). Additionally, there are some reports of changes in metal metabolism

during molt (Martin, 1975; Adams *et al.*, 1982) which may involve the metal-binding protein metallothionein.

Metallothionein has been characterized as a detoxifying protein that is synthesized in response to elevated metal concentrations both internally and in the environment. It is a low molecular weight protein (<10,000 M_r) which binds cadmium, copper, zinc, and mercury, and contains high concentrations of cysteine (>20%) (Cherian and Goyer, 1978). Since initial isolation by Margoshes and Vallee (1957), metallothioneins have been studied extensively in mammals and invertebrates (Kojima and Kagi, 1978; Nordberg and Kojima, 1979; Roesijadi, 1981). These investigations have been concerned primarily with the involvement of metallothionein in detoxification of metals in animals and with the characterization of the protein molecule. Currently there is renewed interest in determining "normal" function of metallothioneins and the metabolic processes that control constitutive levels of metallothioneins in unstressed organisms (Engel and Brouwer, 1984a; Cousins, 1985; George and Viarengo, 1985; Engel and Roesijadi, in press).

Metallothioneins have been demonstrated in marine crustaceans exposed to elevated levels of cadmium and copper in their water or food (Olafson *et al.*, 1979a, b; Overnell and Trehwella, 1979; Rainbow and Scott, 1979; Overnell, 1982; Wiedow *et al.*, 1982; Engel and Brouwer, 1984b; Engel *et al.*, 1985; Engel and Brouwer, 1986; Overnell, 1986). In the blue crab, metallothioneins have been shown to bind cadmium, copper, and zinc in both laboratory exposed animals (Brouwer *et al.*, 1984; Engel *et al.*, 1985) and in animals from contaminated environments (Weidow *et al.*, 1982; Engel and Brouwer, 1984b). Gel chromatography of the digestive gland cytosol yields proteins that have an estimated molecular weight of 7,000–10,000 M_r and bind the three metals. Further purification of these proteins by ion exchange chromatography demonstrated that there was one protein that binds cadmium and zinc and another that binds copper. Further characterization of the cadmium metallothionein showed that it had the characteristics of the mammalian metallothionein (Brouwer *et al.*, 1984). In our investigations with blue crab metallothioneins, we have observed apparent correlations between the physiological condition of the crab and the metal distribution in the cytosol.

The present research effort was designed to investigate whether molting contributes to variability in metallothionein concentration and metal composition in blue crabs. These experiments were designed to examine total copper and zinc in the hemolymph and digestive glands and the cytosolic distributions of copper and zinc in the digestive glands of crabs during six different stages of the molt cycle.

MATERIALS AND METHODS

Animals

All blue crabs used in these experiments were collected from estuarine waters in the vicinity of Beaufort, North Carolina. Three groups of crabs were obtained throughout the summer of 1985 and these animals included premolt, soft, papershell, and intermolt crabs, and were comprised of about 75% immature females. The temperature and salinity at the time of collection was 20 to 22°C and 31 to 33‰.

The designations of molt stage were according to those described by Mangum (1985) for the blue crab and by Passano (1960) for brachyuran molt stages.

Tissue metal measurements

The concentrations of copper and zinc were determined in samples of digestive gland and hemolymph from individual blue crabs. Hemolymph samples were col-

lected by severing the fifth pereopod at the meropodite and collecting the fluid in a polyethylene vial. A portion of the hemolymph was taken for metal analysis and the remainder was used for determination of hemocyanin concentration. The crabs were killed by removing the carapace, and the digestive gland was dissected and used for total metal measurements and cytosolic metal determinations. The tissue used for determination of cytosolic distribution of metals was flash-frozen and stored in a freezer at -70°C .

Tissue samples used for metal analysis were oven dried at 100°C for 48 hours and wet ashed with concentrated HNO_3 at 90°C . Residue was dissolved in 0.25 N HCl , and concentrations of copper and zinc were measured using flame atomic absorption spectrophotometry. Preparative and measurement techniques were calibrated against the National Bureau of Standards Oyster Reference Material #1566.

Measurements of hemocyanin concentrations in the blue crab hemolymph samples were made by Dr. Marius Brouwer (Duke University Marine Laboratory/Bio-medical Center, Beaufort, North Carolina). The clotted hemolymph was homogenized, centrifuged, and hemocyanin concentration was determined spectrophotometrically using the extinction coefficient of the native undissociated protein (Johnson *et al.*, 1984).

Chromatography

Individual crab digestive glands were used in each chromatographic separation. Frozen tissue samples were weighed and then placed in 10 ml of 60 mM Tris buffer, pH 7.9, with $2\text{ mM 2-mercaptoethanol}$ in an ice bath and homogenized 30 seconds with Brinkman Polytron¹ homogenizer. The homogenate was then centrifuged at $105,000 \times g$ for 90 minutes at 4°C . The resulting supernate was considered to be the cytosolic fraction. Mass balances were conducted on each sample, and aliquots of the homogenate and cytosol and the entire pellet with fat cap were analyzed for metals according to the procedure outlined above.

Gel-chromatography of the cytosolic fraction was done on a Sephadex G-75 column ($2.6\text{ cm} \times 66\text{ cm}$). The elution buffer was 60 mM Tris , pH 7.9, with $2\text{ mM 2-mercaptoethanol}$. Individual 7-ml fractions were analyzed for absorbance at 250 nm, and for concentrations of copper and zinc were measured directly by flame atomic absorption spectrophotometry. The column was standardized with blue dextran ($>10^6\text{ M}_r$), bovine serum albumin ($67,000\text{ M}_r$), ovalbumin ($43,000\text{ M}_r$), chymotrypsinogen A ($25,000\text{ M}_r$), ribonuclease ($13,700\text{ M}_r$), and potassium. The determination of molecular weight was calculated from:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

The elution characteristics of the column were: V_e —the elution volume of an unknown or standard, V_0 —the elution volume of the high molecular weight material excluded from the column, and V_t —the total elution volume of the ionic component or "salt fraction" as measured by potassium. The peak which eluted with an apparent molecular weight of $7,000\text{--}10,000\text{ M}_r$, was considered to be the metallothionein fraction.

Further analysis of the metallothionein peak was performed on a DEAE Sephacel ion-exchange column ($1.6 \times 10\text{ cm}$). A gradient was generated from 250 ml, 60 mM

¹ Mention of a trade name does not constitute an endorsement by the National Marine Fisheries Service, NOAA.

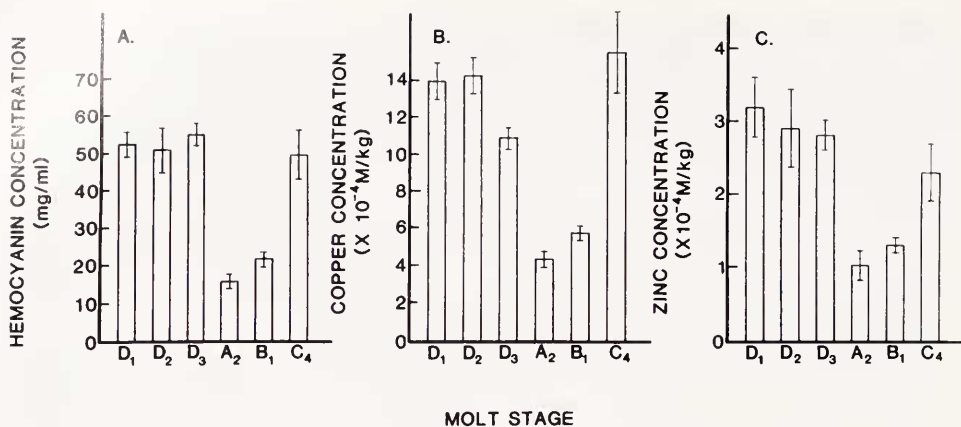


FIGURE 1. Hemolymph concentrations of hemocyanin (A), copper (B), and zinc (C), from blue crabs during different stages of the molt cycle. Molt cycle stages were: premolt white sign (D₁), pink sign (D₂), and red sign (D₃); soft shell (A₂); paper shell (B₁); and intermolt (C₄). Hemocyanin measurements made by Dr. M. Brouwer, Duke Marine Biomedical Center, Beaufort, NC. Each mean represents five or six individual hemolymph samples \pm 1 standard error.

Tris, pH 7.9, and 250 ml, 300 mM Tris, pH 7.9, both contained 2 mM 2-mercaptoethanol. Fractions were analyzed for conductivity and concentrations of copper and zinc.

RESULTS

Hemolymph and tissue metal concentrations

Blue crab hemolymph concentrations of hemocyanin, copper, and zinc changed during the molt cycle. Hemocyanin concentrations during the soft crab (A₂) and papershell (B₁) stages were significantly different ($P < 0.05$) from the three premolt stages (D₁, D₂, D₃) (Fig. 1A). The concentrations of copper and zinc followed the same pattern as the hemocyanin and also were different from the intermolt crabs (C₄) (Fig. 1B, C). The correlation between copper and hemocyanin would be expected, since copper is an integral part of the hemocyanin molecule. The positive correlation between changes in hemocyanin concentration and zinc concentrations suggests that the zinc may be bound to hemocyanin (Fig. 1C).

The digestive gland concentrations of copper and zinc were reflective of the physiological changes that occur during the molt cycle in the blue crab. Copper concentrations in the digestive gland showed significant decreases ($P < 0.05$) in both the soft and papershell stages (Fig. 2A), and the papershell stage also was lower than the soft crab stage ($P < 0.05$). The differences were 3.4 and 6.0 times lower than the D₁ stage of premolt. The observed decreases in copper in the digestive gland are in close correlation with decreases of copper in the hemolymph (Fig. 1B). This means that copper is being lost or excreted, and not conserved by the digestive gland. In the initial portions of the molt cycle, however, it appears that, as the crabs move from intermolt (C₄) to premolt (D₁-D₃), there is an increase in copper in the digestive gland, which may be associated with changes in hemocyanin synthesis and degradation, though this retention of copper does not persist. Zinc concentrations showed the same type of variation during the intermolt and premolt periods as copper, and the soft and

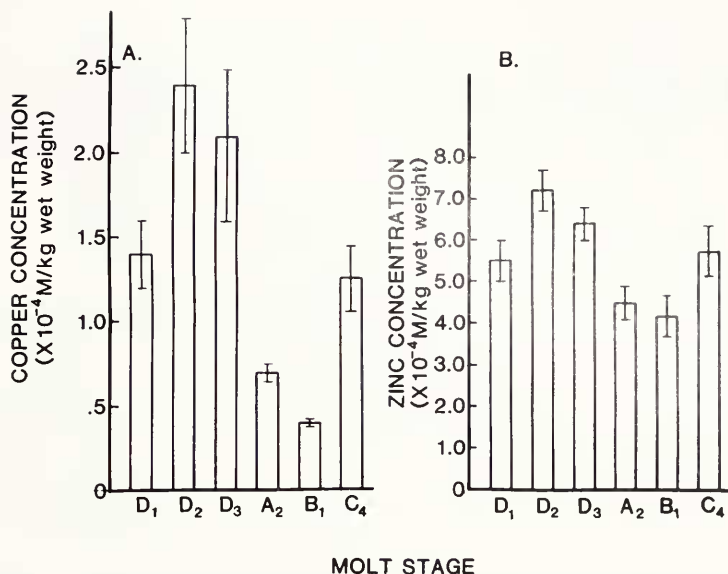


FIGURE 2. Digestive gland concentrations of copper (A) and zinc (B) from blue crabs during different stages of the molt cycle (see Fig. 1). All mean values represent five or six individual samples of digestive gland \pm 1 standard error.

papershell stages also were different from premolt crabs (D₂), ($P < 0.05$) (Fig. 2B). The concentrations of copper and zinc in the digestive glands of the C₄ and D₁ stage crabs were in good agreement with concentrations measured previously in intermolt blue crabs collected near Beaufort (Engel and Brouwer, 1984b).

Cytosolic metal distribution

The Sephadex G-75 elution profile for the cytosol prepared from the digestive gland of a D₁ stage blue crab showed partitioning of the metals with a pronounced 7–10 K M_r metallothionein-like protein that contained zinc and copper (Fig. 3A). Both zinc and copper also were present in the high molecular weight material (fractions 13–22), but only zinc was present in the very low molecular weight material (fractions 42–52).

Further characterization of the metallothionein-like protein peak (fractions 32–38) was done on a DEAE Sephacel ion-exchange column with a Tris gradient 60–300 mM Tris, pH 7.9. Three metal containing peaks were resolved, a zinc component in the breakthrough volume and copper and zinc components that eluted at conductivities of 5.5 and 6.0 mS/cm (Fig. 3B). These conductivities represent Tris concentrations of 110 and 125 mM Tris at pH 7.9, which compare favorably to the data of Brouwer and co-workers (Duke University Marine Biomedical Center, Beaufort, NC, pers. comm.) for cadmium metallothionein in blue crabs. Therefore, the protein peak eluting from the G-75 gel filtration column at an apparent molecular weight of 7–10 K M_r will be referred to as metallothionein (MT). The observed separation between the copper and zinc peaks, however, does not necessarily mean that they are separate copper and zinc-binding proteins, but may simply reflect differences in charge characteristics on the protein.

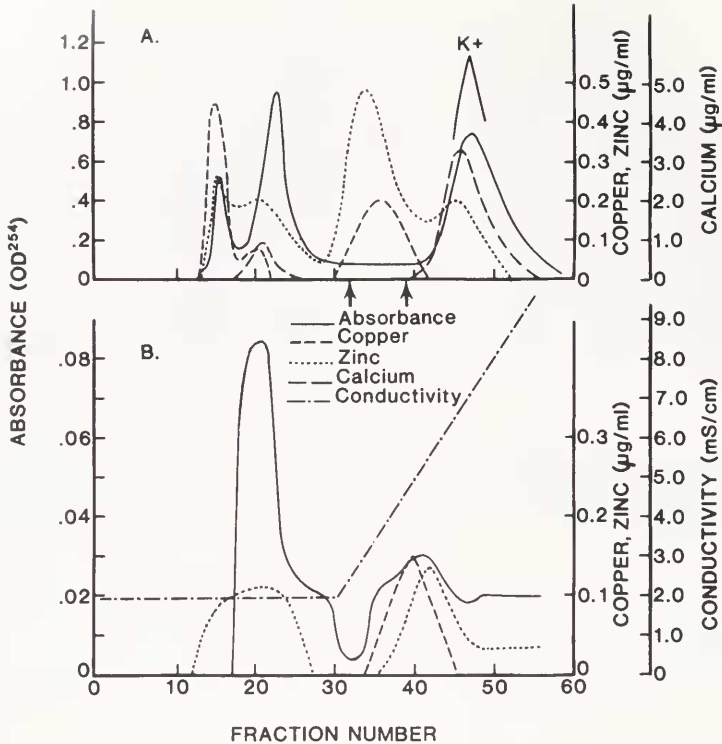


FIGURE 3. Elution profile of the cytosolic fraction of the digestive gland of an intermolt crab showing the partitioning of metals; (A) Sephadex G-75 elution profile column 2.6×65 cm with a flow rate of 30 ml/h of 60 mM Tris, pH 7.9 with 2 mM-2 mercaptoethanol and (B) DEAE—Sephacel ion-exchange column 1.6×10 cm using a gradient generate from 250 ml, 60 mM Tris, pH 7.9 and 250 ml, 300 mM Tris, pH 7.9 and both contained 2 mM-2 mercaptoethanol. The arrows denote the metallothionein peak that was pooled and subjected to ion exchange chromatography. The peak marked K^+ represents the position of the potassium peak (very low molecular weight).

The individual elution profiles of digestive gland cytosol for the six different stages of molting blue crabs show differences in metal distributions that can be correlated with the molting process (Fig. 4). The premolt crabs examined (D_1 , D_2 , D_3) have a very pronounced metallothionein peak, which is dominated by zinc (Fig. 4A, B, C). Zinc is also present in the high (>60 K, M_r), intermediate (~ 40 K, M_r), and very low (<1 K, M_r) molecular weight fractions. Copper is present only in the high and metallothionein portions of the profile. In the soft crabs (A_2), both zinc and copper are present in the metallothionein region (Fig. 4D), and the relative amount of copper has increased. Otherwise, the distributions of copper and zinc in the elution profiles are the same as before. The metal content of the papershell crabs (B_1) is entirely copper with no measurable zinc (Fig. 4E), and the zinc is present only in the high, intermediate, and low molecular weight fractions. Hard shell or intermolt crabs (C_4) have a very elevated copper metallothionein with a low amount of zinc present in the same fractions (Fig. 4F), and zinc follow the same pattern as seen in the A_2 and B_1 crabs.

Estimates of total copper/zinc binding proteins

Calculations to estimate the total metallothionein bound metal in the digestive gland cytosol of the different molt stages were made using the modified relationship of Frazier and George (1983). The relationship is as follows:

$$\text{B.P.} = \frac{[\text{Zn}]}{6} + \frac{[\text{Cu}]}{10} \left/ \frac{\text{Adjusted Sample Volume}}{(10 \text{ ml} = 100\%)} \right/ \frac{\text{Wet Weight of Tissue}}{\text{in grams}}$$

The molar concentrations of copper and zinc were calculated from the five peak fractions of each metallothionein peak and were adjusted for applied sample volume and wet weight of tissue. It was assumed that individual metallothionein molecules bind only copper or zinc, and that the blue crab metallothionein binds six zinc atoms [e.g., the *Scylla* protein binds 6 moles of cadmium per mole of protein which is assumed to be the same as zinc (Olafson *et al.*, 1979)], or 10 atoms of copper [e.g., conservative estimate from conversations with M. Brouwer (pers. comm)]. Such calculations are estimations of concentration and are not meant to be quantitative, but do allow for comparisons between groups of crabs.

When the comparisons were made between the different groups of crabs, differences not only were observed in the total amount of protein-bound metal, but also in the relative amount of copper and zinc that was present (Fig. 5). The estimated concentrations of protein- (*i.e.*, metallothionein) bound metal followed roughly the same pattern as total copper and zinc concentrations in tissue (Fig. 5A). When the percentages of metallothionein-bound copper were calculated, they ranged from 7% to 10% of total in the premolt stages, to 35% in the soft crab, to 100% in the paper shell, and 75% at intermolt (Fig. 5B). Such differences show that the cytosolic distributions of metals change during molting and that metal metabolism and metallothionein must be linked directly with the extensive cellular processes that are occurring. This hypothesis is supported further by the observed changes in the percentages of total digestive gland copper and zinc that are present in the cytosolic fraction (Fig. 5C). These calculations, made from the mass balances, show that the percentages of copper in the cytosol at different stages of molting range from 60 to 80 percent. During A₂ and B₁, when the copper bound to metallothionein is increasing, the percentage of zinc in the cytosol drops from ~75 to 34 percent. This decrease can be accounted for by zinc in the pellet, indicating that there is an intracellular redistribution of metal, which results in some conservation. Since the zinc is in the pellet, it must be incorporated into relatively inert structures or bound to membranes that were not disrupted by the homogenizing process.

DISCUSSION

The blue crabs used in these experiments were not exposed to artificially elevated concentrations of metals in the laboratory. Therefore, changes in metal concentrations in the hemolymph and the digestive gland and in cytosolic metal distributions were constitutive processes associated with molting. If it is assumed that these changes in metal partitioning are involved in the synthesis of metalloproteins, such as enzymes and respiratory proteins, then a model of metal metabolism and metallothionein function for blue crabs can be developed.

The greatest changes in overall metal concentration and cytosolic distribution of metals occurred at or around the time of ecdysis and early soft crab. At this time,

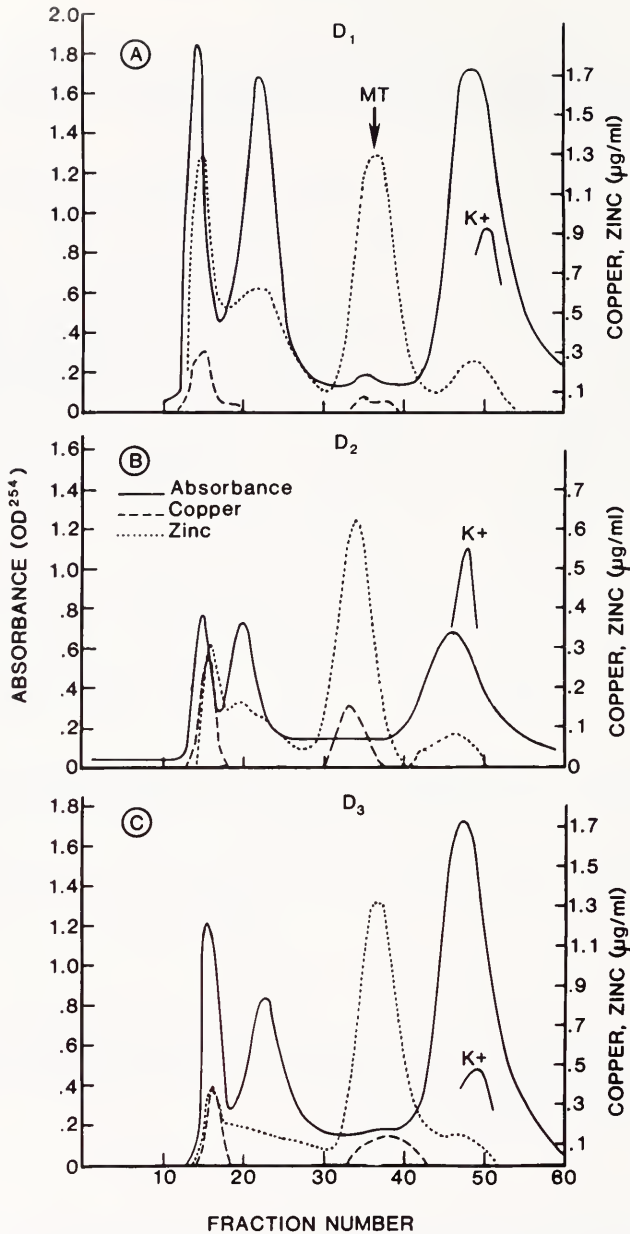


FIGURE 4. A series of representative G-75 elution profiles of digestive gland cytosol from all of the different molt stages that were examined [A(D₁), B(D₂), C(D₃), D(A₂), E(B₁), and F(C₄)]. Conditions were the same as in Figure 3A.

there were significant decreases in the hemolymph concentrations of copper and zinc, which were correlated with decreases in hemocyanin concentrations. These changes in hemocyanin concentration agree favorably with the data of Mangum *et al.* (1985).

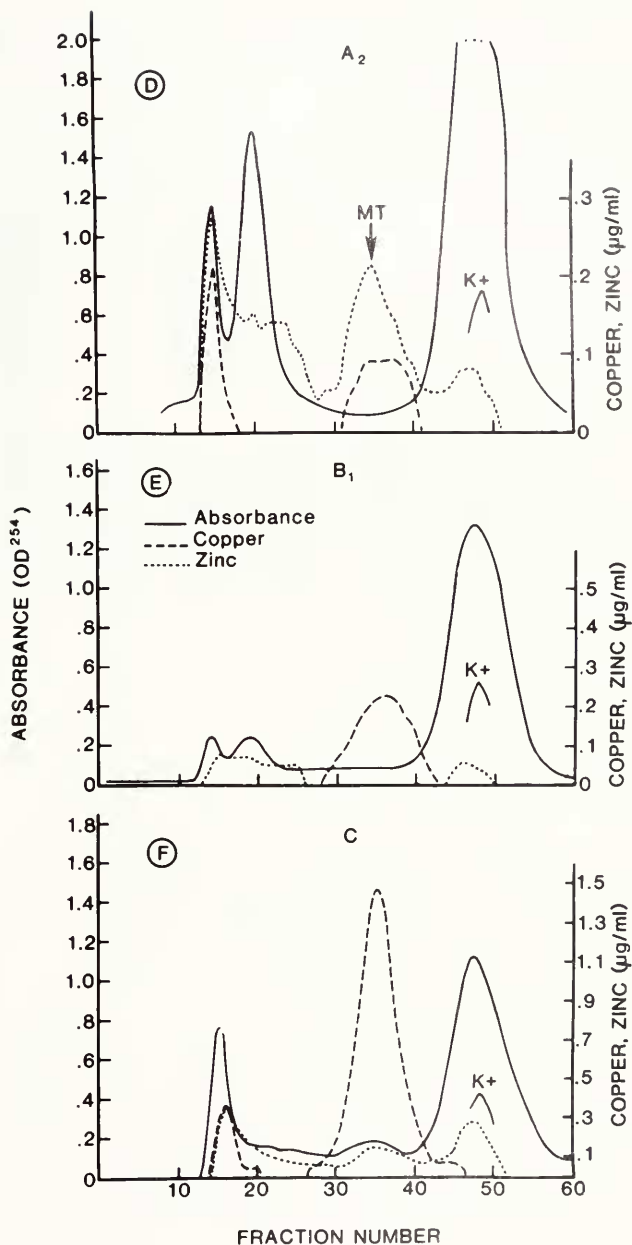


FIGURE 4. (Continued)

The decrease in copper is understandable, since hemocyanin is a copper protein, but the associated decrease of zinc strongly suggests that it also is bound to hemocyanin. Zatta (1984) demonstrated with *Carcinus maenas* that each minimal functional sub-unit of hemocyanin has four zinc-binding sites, and that about 90% of the zinc in the hemolymph was not dialyzable. Brouwer and Engel (1982) also suggested that

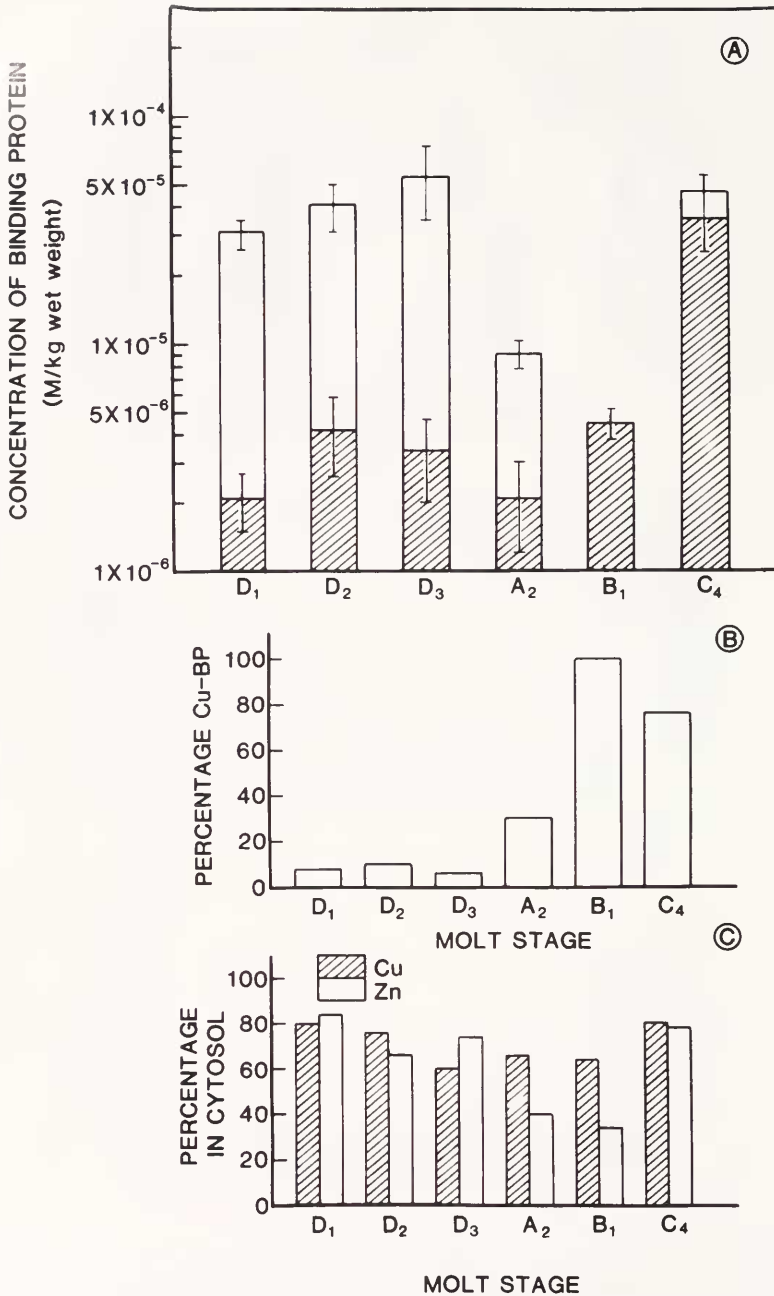


FIGURE 5. Copper and zinc partitioning on the metal-binding protein (*i.e.*, metallothioneins) and in the cytosol of the digestive glands of molting blue crabs. (A) Calculated estimates of total binding protein concentrations (clear bar) and copper-binding protein (cross hatched areas). Means \pm 1 standard error ($n = 5$). (B) Calculated percentage of the total binding protein that binds copper. (C) Percentage of total digestive gland copper and zinc that was present in the cytosol as determined from mass balances on individual digestive glands.

hemocyanin is a major vehicle for the transport of zinc in blue crabs. Additional evidence for the association of zinc with hemocyanin in these experiments comes from the close correlation between the increase in both zinc and hemocyanin in the hemolymph of papershell crabs.

Observed changes in total copper and zinc in the digestive gland during the molt cycle parallel fluctuations in the hemolymph, and the cytosolic distributions of these metals also show large changes. On a molar basis the changes in copper and zinc in the digestive gland between D₃ and B₁, are quite similar (Fig. 2) and suggest that there is some linkage between the two metals. This linkage also is associated with the changes in the cytosolic distributions of these metals. The most pronounced changes in cytosolic distribution, however, occur between premolt and papershell where the predominant metal bound to metallothionein changes from zinc to copper. These events are relatively rapid and are associated with other physiological and biochemical events.

During this portion of the molt cycle the percentage of copper bound to metallothionein increased as the crabs moved toward papershell (Fig. 5B). The percentage of copper associated with the metallothionein peak changed from 10% copper immediately prior to shedding, to virtually 100% copper at the papershell stage. It appears, therefore, that metallothionein changes from primarily binding zinc to binding copper at the time when the newly molted crabs may be resynthesizing hemocyanin. This correlation supports the hypothesis of Brouwer *et al.* (1986) that copper metallothionein is necessary as Cu⁺¹ donor in the synthesis of hemocyanin in blue crabs and lobsters.

Dominance of the copper form of metallothionein continues into the intermolt period and its concentration is high relative to the papershells, but roughly comparable to the total concentrations observed during premolt (Fig. 5A). Even though these animals are stable with regard to molting, the predominance of copper metallothionein suggests a very active turnover of the hemocyanin in the hemolymph of the blue crabs. Senkebil and Wriston (1981) reported that the T_{1/2} for hemocyanin in the lobster was 25.5 days. Since blue crabs may have a higher metabolic rate, their hemocyanin turnover rate may be more rapid. Such turnover rates would require an active pool of Cu⁺¹ for degradation, storage, and synthesis of hemocyanin.

In the molt cycle of the blue crab, there are significant decreases in copper in both the hemolymph and digestive gland at the time of molting (Fig. 1B and 2A). Since it appears that the copper is lost by the animal upon molt, portions of the exuvia were measured for copper. These measurements did not show elevated levels of copper in either the molted exoskeleton or stomach and gut linings. Therefore, we do not know the route of copper excretion in the blue crab at molt. Djangmah (1970) indicated that *Crangon vulgaris* excreted copper during molt, and must accumulate it again from either water or food. Such large changes in copper in both hemolymph and the digestive gland also have been demonstrated in the crab, *Cancer irroratus* (Martin, 1975), and in the crayfish, *Austropotamobius pallipes* (Adams *et al.*, 1982). Pathways of metal loss have been examined recently in the shrimp, *Penaeus semisulcatus*, (Al-Mohanna and Nott, in press), using both atomic absorption spectrophotometry and electronmicroscopy with EDAX. They demonstrated that copper and zinc fluctuated during the molt cycle, and that membrane-bound intracellular inclusions were present in cells of the hepatopancreas. These granule-containing cells appear to be sloughed at molt, and the granules which are rich in both copper and sulfur may relate back to metallothionein, and constitute a pathway of excretion for metallothionein bound metals.

While it is possible to relate copper metallothionein to hemocyanin synthesis, it is more difficult to relate zinc metallothionein concentration to any specific synthetic process in the crabs. During the premolt period D₁-D₃ the predominance of zinc metallothionein is clear, with 90-94% of the total consisting of zinc metallothionein. The decrease in copper metallothionein is probably associated with a decrease in hemocyanin synthesis. Since it is during the premolt period that the new epidermal material is produced, the mobilization of zinc probably correlates with increased need for zinc-dependent enzymes (*i.e.*, carbonic anhydrase) associated with calcification in the new exoskeleton (Henry and Kormanik, 1985). Unlike copper, which varies in concentration in the digestive gland depending upon the environmental conditions, zinc remains remarkably stable (Engel and Brouwer, 1984b). Additional data on tissue concentration of zinc in digestive glands of blue crabs from Long Island Sound to Florida further establish the fact that zinc is highly regulated by blue crabs (D. W. Engel, unpub. data). Also during the molt process, the copper concentration varies by a factor of 6 in the digestive gland, but zinc varies only by a factor of 1.7 (Fig. 2). The absolute changes on a molar basis are similar which suggests a linkage in metabolism.

The data presented here may alter the perception of metallothionein as primarily a detoxifying protein. The function of metallothionein may be one of a regulatory protein for copper and zinc metabolism in unperturbed crabs. Since most of the early research emphasis was on its ability to bind cadmium and mercury, both toxic metals, it is not surprising that the focus was on detoxification. Cousins (1985), however, discusses some of the functions of metallothionein and indicates that it is also involved in stress reactions. Udom and Brady (1980) and Li *et al.* (1980) demonstrated that mammalian zinc metallothionein has the capability of reactivating apo-carbonic anhydrase, and they suggest that metallothionein has a regulatory or transport function in normal physiology. Brouwer *et al.* (1986) has hypothesized that copper metallothionein is the Cu⁺¹ donor for the synthesis of crustacean hemocyanin, and the results of this investigation support that hypothesis. They also go a step further in that they show that the pathways of zinc and copper, while linked through their binding to metallothionein, follow different pathways of mobilization and turnover.

The present studies have established that normal physiological processes, such as growth, can influence metallothionein in regard to metal content and concentration in the blue crab. Further, they show that generalized statements with regard to metal metabolism, even within phylogenetic groups, may not be valid, as shown by our data on blue crabs and lobsters. Finally, the present investigation demonstrates the need for basic physiological and biochemical information in order to understand the processes involved in toxicological assessments of environmental impacts of metals.

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